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Increasing MuSK Activity Delays Denervation and Improves Motor Function in ALS Mice

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SUMMARY

Amyotrophic lateral sclerosis (ALS) is a devastating disease that progresses from detachment of motor nerve terminals to complete muscle paralysis and lethal respiratory failure within 5 years of diagnosis. Genetic studies have linked mutations in several genes to ALS, and mice bearing mutations in *SOD1* recapitulate hallmark features of the disease. We investigated whether disease symptoms can be ameliorated by co-opting the retrograde signaling pathway that promotes attachment of nerve terminals to muscle. We crossed *SOD1G93A* mice with transgenic mice that express MuSK, a receptor tyrosine kinase that is required for retrograde signaling, and we used histological and behavioral assays to assess motor innervation and behavior. A 3-fold increase in *MuSK* expression delayed the onset and reduced the extent of muscle denervation, improving motor function for more than a month without altering survival. These findings suggest that increasing MuSK activity by pharmacological means has the potential to improve motor function in ALS.

INTRODUCTION

The withdrawal of motor axons from muscle is the first sign of disease in familial and sporadic forms of amyotrophic lateral sclerosis (ALS), portending a progressive and devastating loss of motor function that culminates in lethal muscle paralysis within 5 years of diagnosis (Fischer et al., 2004; Pasinelli and Brown, 2006; Schaefer et al., 2005). The mechanisms responsible for axon withdrawal are poorly understood, but the loss of neuromuscular synapses is sufficient to cause muscle paralysis and therefore is central to the disease. Although the subsequent loss of motor neurons has received more attention, preventing or delaying motor neuron cell death without preserving neuromuscular synapses cannot stop disease progression. Moreover, methods designed to inhibit motor neuron cell death, either by blocking cell death pathways or by providing broadly acting growth factors, have had only a modest impact on disease progression (Kostic et al., 1997; Pun et al., 2006; Sagot et al., 1995).

Dominant mutations in *SOD1*, *TDP-43*, *FUS*, and the recently discovered *C9orf72* gene are responsible for familial forms of ALS and together represent ~17% of all cases of ALS (Chen-Plotkin et al., 2010; Kwiatkowski et al., 2009; Majounie et al., 2012; Pasinelli and Brown, 2006; Renton et al., 2011; Vance et al., 2009). The pathological hallmarks of the disease are well replicated in mice that overexpress mutant forms of SOD1 in motor neurons, and therefore such mice provide an excellent model system for studying the pathology of ALS and identifying approaches to treat this devastating disease (Pasinelli and Brown, 2006). Although cell types other than motor neurons, including gray matter oligodendrocytes and microglia, contribute to disease onset and progression (Ilieva et al., 2009; Kang et al., 2010), dominant mutations in *SOD1* act largely in an autonomous manner within motor neurons, consistent with the idea that ALS is primarily, though not entirely, a disease of upper and lower motor neurons.

Skeletal muscles provide retrograde signals that promote the differentiation and stabilization of motor nerve terminals (Burden, 1998; Sanes and Lichtman, 2001). In the absence of these muscle-derived retrograde signals, developing motor axons grow aimlessly within muscle and fail to form synapses. The production of muscle-derived retrograde signals depends upon a synaptic receptor tyrosine kinase, termed MuSK, and Lrp4, a receptor for Agrin that forms a complex with MuSK (DeChiara et al., 1996; Kim et al., 2008; Weatherbee et al., 2006; Zhang et al., 2008; Yumoto et al., 2012). During normal development, Lrp4/MuSK signaling initiates neuromuscular synapse formation, whereas the subsequent stabilization of nascent synapses also requires neuronal Agrin, which binds to Lrp4 and strongly stimulates MuSK (Kim and Burden, 2008; Zhang et al., 2008, 2011). In the absence of Agrin, neuromuscular synapses form, but motor axon terminals subsequently withdraw, leading to defective neuromuscular transmission and perinatal death (Gautam et al., 1996; Lin et al., 2001, 2005; Misgeld et al., 2005). Retrograde signaling also regulates the stability and maintenance of synapses in adult animals, as interfering with MuSK function in adult mice causes disassembly of neuromuscular synapses (Hesser et al., 2006; Kong et al., 2004). Because a failure to maintain neuromuscular synapses is central to all forms of ALS, we tested whether increasing retrograde signaling in *SOD1* transgenic mice could stabilize neuromuscular synapses, delay axon withdrawal, and ameliorate the symptoms of disease.

RESULTS

Previously, we found that a modest (3-fold) increase in *MuSK* expression is sufficient to maintain neuromuscular synapses in

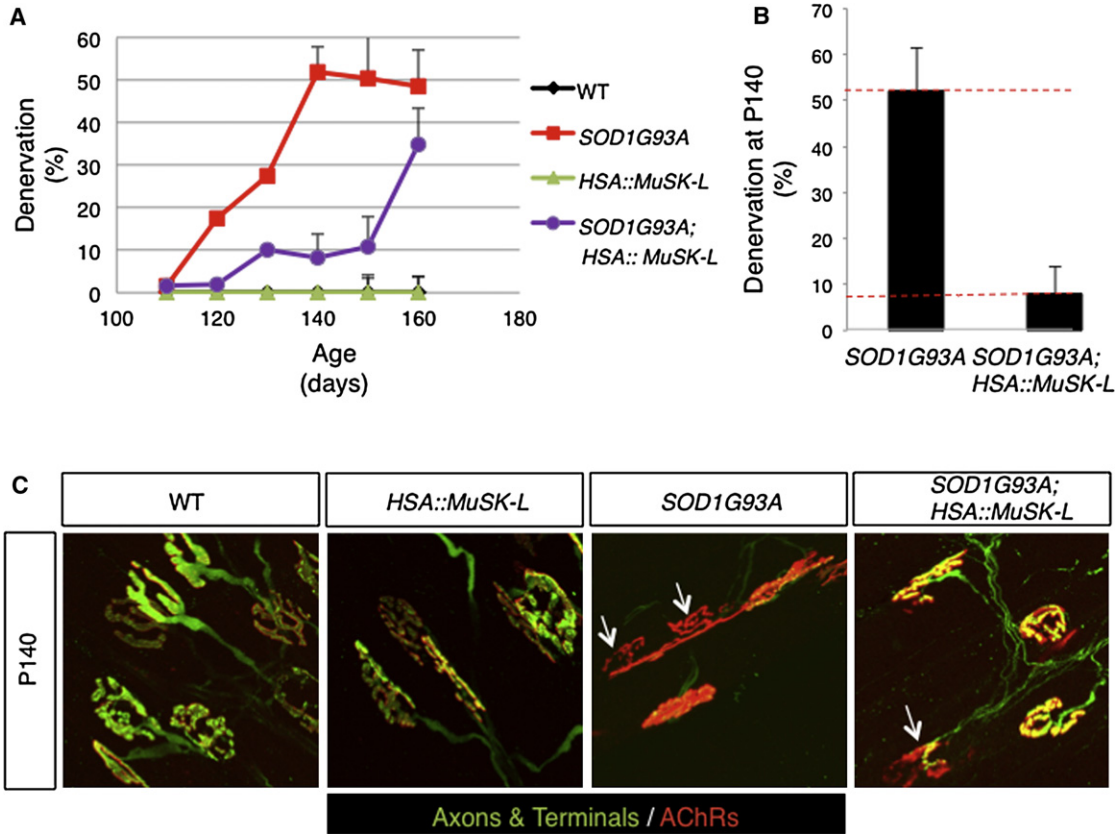

Figure 1. *MuSK* Overexpression Delays the Onset and Reduces the Extent of Denervation in *SOD1G93A* Mice

(A) Denervation becomes evident at P120 in *SOD1G93A* mice and at P130 in *SOD1G93A* mice overexpressing *MuSK*. The extent of denervation remains lower in mice overexpressing *MuSK* through P150.

(B) At P140, only 8% of synapses are denervated in *SOD1G93A* mice overexpressing *MuSK*, whereas 52% of synapses are denervated in *SOD1G93A* mice.

(C) Images of synapses from P140 mice stained with α-BGT to label AChRs and antibodies to NF and Syn to label motor axons and nerve terminals. Staining for Syn and AChRs overlap at innervated synapses, whereas Syn staining is absent from denervated synapses, which retain a high density of AChRs. Error bars are the SEM.

See also Figures S1 and S2.

agrin mutant mice, thereby preventing perinatal lethality and promoting postnatal survival (Kim and Burden, 2008). We therefore wondered whether increasing *MuSK* expression in a mouse model of ALS would stabilize neuromuscular synapses, delay motor axon withdrawal, and increase muscle function.

We crossed *HSA::MuSK-L* mice, which express 3-fold more *MuSK* than wild-type (WT) mice, with *SOD1G93A* mice, and used histological assays to compare the rate and extent of denervation of the *SOD1G93A* and *MuSK-L*; *SOD1G93A* mice. We stained whole mounts of the diaphragm muscle with antibodies against synapsin (Syn) to label nerve terminals and with α-bungarotoxin (α-BGT) to visualize acetylcholine receptors (AChRs) in muscle. In WT and *MuSK-L* transgenic mice, nerve terminals are apposed to AChRs, and the coincidence of Syn/AChR staining defines innervated synaptic sites (Figure 1). In *SOD1G93A* mice, axons from fast, fatigable motor neurons withdraw early in disease (Pun et al., 2006). Axons from slow, nonfatigable motor neurons are lost more slowly and compensate by sprouting and temporarily reoccupying denervated synaptic sites on fast myofibers (Pun et al., 2006). We first measured the number of synaptic sites that lacked Syn staining and were therefore completely denervated. In *SOD1G93A* mice, denervation of the diaphragm muscle became evident at postnatal day 120 (P120) and the extent of denervation increased gradually over the next 20 days, reaching a maximum of ~50% at P140 (Figure 1), similar to the time course and extent of denervation observed in other muscles (Schaefer et al., 2005). In contrast, in *SOD1G93A* mice overexpressing MuSK, denervation of the diaphragm muscle began 10 days later, and the extent of denervation remained <10% through P150 (Figure 1). By P160, the extent of denervation increased and approached the level found in *SOD1G93A* mice (Figures 1 and S1). These findings indicate that *MuSK* overexpression protected synapses in *SOD1G93A* mice from denervation by delaying the onset and decreasing the extent of denervation for >40 days.

The remaining synapses included normally and partially innervated synapses. At partially innervated synapses, Syn staining incompletely overlapped the AChR-rich postsynaptic membrane, leaving patches of the postsynaptic membrane devoid of innervation (Figure 2). To determine whether *MuSK* expression

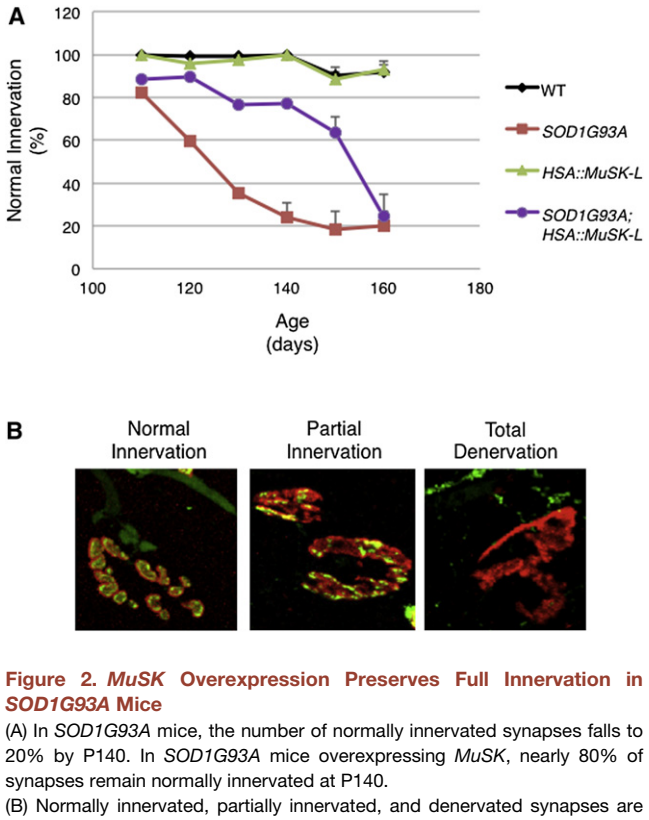

Figure 2. *MuSK* Overexpression Preserves Full Innervation in *SOD1G93A* Mice

(A) In *SOD1G93A* mice, the number of normally innervated synapses falls to 20% by P140. In *SOD1G93A* mice overexpressing *MuSK*, nearly 80% of synapses remain normally innervated at P140.

(B) Normally innervated, partially innervated, and denervated synapses are distinguished by differences in overlap of staining for Syn and AChRs. Error bars are the SEM.

See also Figures S1 and S2.

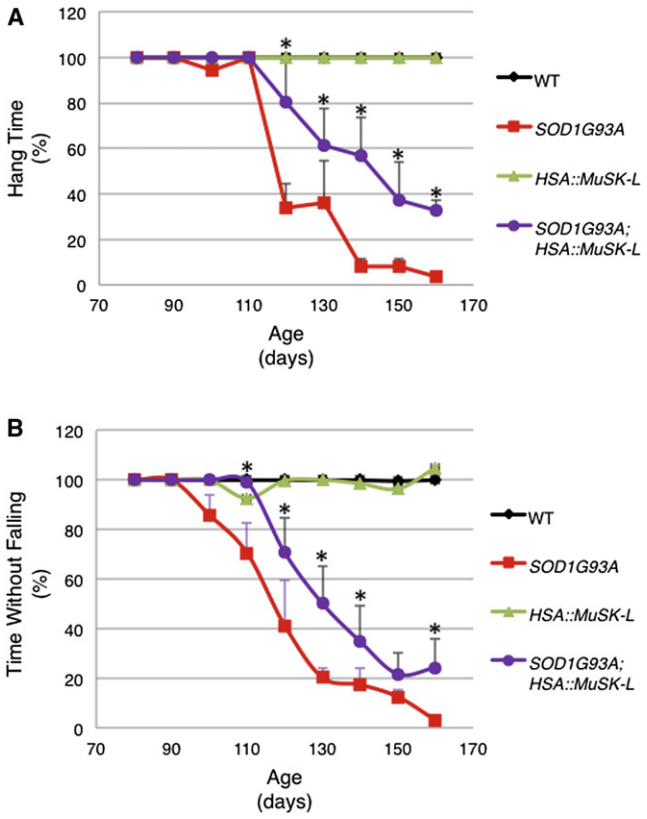

Figure 3. Grip Strength and Rota Rod Performance Are Enhanced in *SOD1G93A* Mice Overexpressing MuSK

(A) The ability of *SOD1G93A* mice to cling to the wire grid declines rapidly after P110. *MuSK* overexpression increases the length of time the mice cling to the wire grid.

(B) *SOD1G93A* mice fall from the Rota Rod beginning at P100, whereas mice overexpressing *MuSK* begin to fall at P120. *SOD1G93A* mice overexpressing *MuSK* remain on the rotating bar longer than the *SOD1G93A* mice at all subsequent times. We analyzed 25–30 mice at P80–P120, 35 mice at P130, 40 mice at P140, and 32 mice at P150 and 32 at P160. Significantly different values ($p < 0.05$) for *SOD1G93A* and *SOD1G93A; HSA::MuSK-L* mice are indicated (*). Error bars are the SEM.

See also Figure S2 and Movie S1.

preserved normal innervation, we measured the number of synaptic sites that were normally innervated. Figure 2 shows that the number of normally innervated synapses fell dramatically after P110 in *SOD1G93A* mice, reaching a plateau of 20% at P140. *MuSK* overexpression in *SOD1G93A* mice preserved normal innervation: the fall in normal innervation was delayed, and >60% of synapses remained normally innervated as late as P150. These findings show that *MuSK* overexpression preserves normally innervated synapses in *SOD1G93A* mice for >40 days. *MuSK* overexpression may preserve innervation by delaying denervation or increasing reinnervation, or both.

To determine whether the delay in onset and decrease in extent of denervation were accompanied by improved motor function, we used a Rota Rod and an inverted grid hanging test to measure the motor performance of the *SOD1G93A* and *MuSK-L*; *SOD1G93A* mice. Figure 3 shows that the *MuSK-L*; *SOD1G93A* mice outperformed the *SOD1G93A* mice in both tests of motor function. The *MuSK-L*; *SOD1G93A* mice clung to the inverted grid longer than the *SOD1G93A* mice, beginning at P120 and continuing through P160, the last time point that we measured. Although the motor function of the *MuSK-L*; *SOD1G93A* mice declined over time, the *SOD1G93A* mice overexpressing MuSK outperformed the *SOD1G93A* mice by 2- to 3-fold at P120 and P130, and by 5- to 9-fold from P140 through P160 (Figure 3A). Likewise, the *MuSK-L*; *SOD1G93A* mice outperformed the *SOD1G93A* mice on the Rota Rod from P100 through P160 (Figure 3B). Moreover, we were able to observe these differences in motor behavior simply by monitoring the mobility of mice in their housing (Movie S1). Nonetheless, the benefit from *MuSK* overexpression is not enduring, as the eventual withdrawal of nerve terminals and decrease in motor function led to death at a time point similar to that observed in the *SOD1G93A* mice (Figure 4A).

DISCUSSION

We show that a modest increase in *MuSK* expression is sufficient to maintain neuromuscular synapses in *SOD1G93A* mice, delaying muscle denervation and improving muscle function for >40 days. These findings indicate that the loss of motor nerve terminals can be delayed by co-opting a retrograde signaling pathway that normally functions to stimulate the differentiation

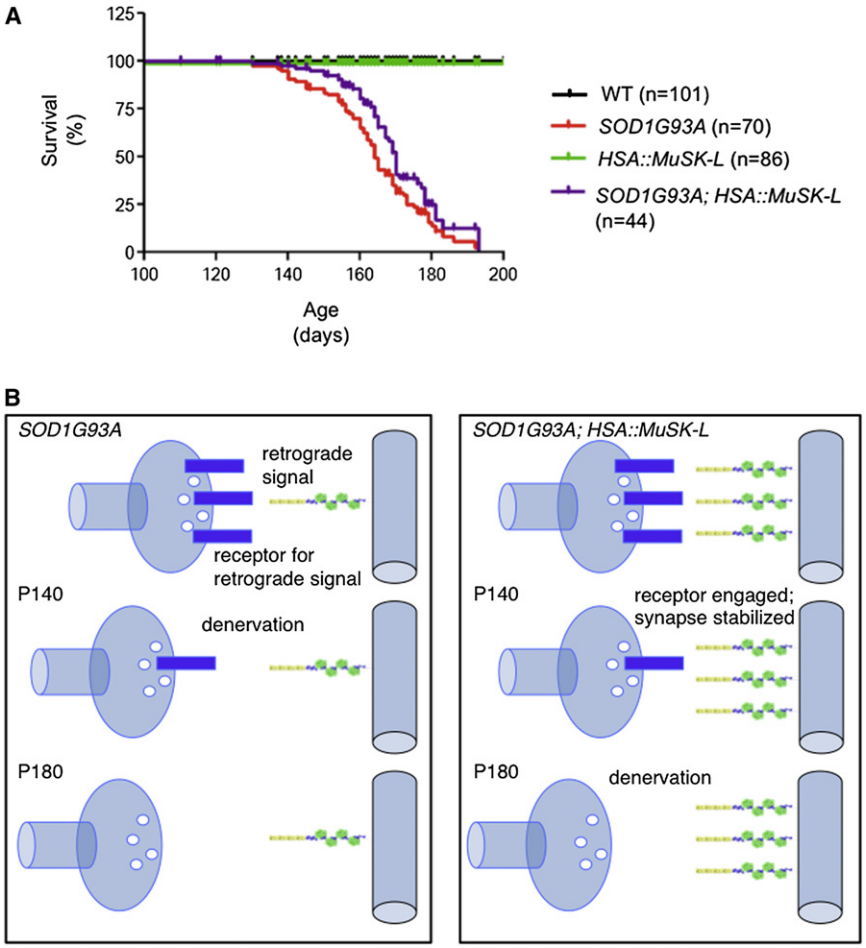

Figure 4. *MuSK* Overexpression Does Not Increase Longevity of *SOD1G93A* Mice

(A) The mean survival of *SOD1G93A; HSA::MuSK-L* mice was 164.82 ± 1.83 days, which is not significantly longer ($p > 0.05$) than that of *SOD1G93A* mice (160.1 ± 2.09 days).

(B) Model for stabilizing motor nerve terminals. In *SOD1G93A* mice, synapses become denervated as motor neurons lose expression of receptors for muscle retrograde signals, which promote differentiation and muscle attachment of nerve terminals. In *SOD1G93A* mice overexpressing *MuSK*, retrograde signaling is enhanced, preserving nerve terminal differentiation and muscle attachment at times when receptor expression is reduced but not absent. Error bars are the SEM.

and stabilization of these terminals. As such, our findings suggest a novel therapeutic approach to slow the steady decline in muscle strength and motor function in ALS. Moreover, because motor axon withdrawal is an early, characteristic, and critical feature of disease in all forms of ALS, we expect that increasing MuSK activity might provide benefit in both the familial and sporadic forms of ALS.

The benefit from *MuSK* overexpression is not permanent. The eventual withdrawal of nerve terminals and loss of motor function indicate that the motor neurons ultimately become so compromised that the motor terminals can no longer be stabilized by increasing MuSK signaling from muscle, suggesting that the terminals eventually lose their ability to respond to critical MuSK-dependent muscle-derived signals. Our recent studies demonstrated that Lrp4 is a critical muscle-derived retrograde signal that acts bidirectionally to coordinate pre- and postsynaptic differentiation (Yumoto et al., 2012). A failure to transport the Lrp4 receptor, or components that act downstream from this receptor, within motor axons may ultimately render motor neurons unresponsive to retrograde signaling (Figure 4B). Identification of the Lrp4 receptor might provide additional targets that can be manipulated to strengthen and prolong the response of compromised motor neurons. Alternatively, the eventual withdrawal of nerve terminals could be caused by the death of motor neurons, a late event in ALS that becomes apparent in *SOD1G93A* mice 1–2 months after denervation has reached a plateau (Fischer et al., 2004). If so, combining therapies that increase MuSK activity with other therapies aimed at promoting motor neuron survival might lengthen the duration of benefit.

As with other receptor tyrosine kinases, tyrosine phosphorylation and activation of MuSK depend upon MuSK dimerization (Stiegler et al., 2009), which is stimulated by binding of Agrin to Lrp4 (Zhang et al., 2011). Although MuSK overexpression is sufficient to promote MuSK dimerization and increase MuSK kinase activity (Watty et al., 2000), it will be necessary to find alternatives to *MuSK* overexpression to activate MuSK in vivo. Soluble forms of neuronal Agrin or Lrp4 may stimulate MuSK in vivo, and a screen for small-molecule activators of MuSK may identify new agonists. Human single-chain variable-region antibodies (ScFv) to MuSK stimulate MuSK tyrosine phosphorylation and AChR clustering in cultured myotubes (Xie et al., 1997). The mechanisms by which these agonist antibodies stimulate MuSK are poorly understood, but they may provide an alternative means to activate MuSK in vivo, an approach that we are currently investigating.

EXPERIMENTAL PROCEDURES

Animals

Mice overexpressing human *SOD1* (B6.Cg-Tg(SOD1-G93A)1Gur/J) were purchased from Jackson Laboratory (Bar Harbor, ME) and crossed with *HSA::MuSK-L* transgenic mice, which were also maintained on a C57BL/6J background (Kim and Burden, 2008). *SOD1G93A* mice were genotyped by PCR (5′-CATCAGCCCTAATCCATCTGA-3′ and 5′-CGCGACTAACAATCAA AGTGA-3′). Primers for IL-2 (5′-CTAGGCCACAGAATTGAAAGATCT-3′ and 5′-GTAGGTGGAAATTCTAGCATCATCC-3′) were used as an internal control.

HSA::MuSK-L transgenic mice express 3-fold more *MuSK* than WT mice (Kim and Burden, 2008). Genotyping was performed by PCR (5′-GAAGC AACCTTTCCTTCCTGAG-3′ and 5′-ATTTTCCCTGAGAGCATTGTCC-3′). All

experiments were approved by the Animal Care and Use Committee of the New York University School of Medicine.

Immunohistochemistry

Diaphragm muscles from adult mice were dissected and fixed for 1.5 hr at room temperature in 1% formaldehyde in phosphate-buffered saline (PBS). Muscles were washed three times for 15 min in PBS, incubated for 15 min with 0.1 M glycine in PBS, and rinsed in PBS and 0.5% Triton X-100 (PBT). Muscles were incubated for 1 hr in PBT containing 4% normal goat serum (PBTG), and overlying connective tissue was diligently removed. Axons and nerve terminals were labeled with rabbit polyclonal antibodies against neurofilament (NF, 1:3,000; Synaptic Systems, Goettingen, Germany) and Syn (1:2,000; Synaptic Systems, Gottingen, Germany) overnight at 4°C in PBTG. After three 1 hr washes in PBT, the muscles were incubated at 4°C overnight with Alexa-488 goat anti-rabbit IgG (1:500; Invitrogen) and Alexa-594-conjugated-α-BGT (1:1,000 in PBTG; Invitrogen, San Diego, CA) to label AChRs. The muscles were washed three times with PBS over 1 hr, postfixed (1% formaldehyde in PBS) for 10 min, rinsed in PBS, and mounted in Vectashield (Vector Labs, Burlingame, CA) under a glass coverslip.

Diaphragm muscles from P90 to P160 WT, *HSA::MuSK-L*, *SOD1G93A*, and *SOD1G93A; HSA::MuSK-L* mice were stained with Alexa-594-conjugated α-BGT and antibodies to NF and Syn. Confocal images of diaphragm muscles were captured on a Zeiss 510 confocal laser scanning microscope (Carl Zeiss MicroImaging, Jena, Germany) using a 40X PlanApo objective. Images were compiled into a reconstructed image, and the number of normally innervated, partially innervated, and fully denervated synapses was quantified. For each experiment, at least 100 synaptic sites were counted for each genotype, and experiments were performed at least three times.

Survival

To measure longevity, we followed the survival of 300 animals (WT, n = 101; *HSA::MuSK-L,* n = 86; *SOD1G93A*, n = 70; and *SOD1G93A; HSA::MuSK-L*, n = 44) for up to 10 months. Kaplan Meier survival curves were generated with the use of GraphPad Prism software.

Behavioral Tests

Motor function was assessed once per week on a Rota Rod (EZ-Rod 3.05; Accuscan Instruments, Columbus, OH). In each experiment we measured the running performance of at least six animals of each genotype from P80 to P160. Mice were placed on a Rota Rod (3.0 cm rotating cylinder) rotating at 1 rpm, and the speed of rotation was gradually increased from 1 to 12 rpm over the course of 40 s and then maintained at 12 rpm for a maximum of 5 min. We recorded the length of time the mice remained on the Rota Rod. WT and *HSA::MuSK-L* mice routinely ran for the full 5 min, yielding an assigned value of 100%; the values for other mice were expressed relative to WT mice.

We assessed motor fatigue using an inverted grid hanging test (Kaja et al., 2007). Individual mice were placed in the center of a wire grid, which was mounted 80 cm above a laboratory bench. After gently inverting the grid, we maintained the grid in an inverted position for a maximum of 60 s and recorded the length of time the mice remained attached to the grid. WT mice routinely remained attached to the grid for the duration and were assigned a value of 100%; the values for other mice were expressed relative to WT mice.

Statistical Analysis

All data are expressed as group means ± standard error of the mean (SEM). For the Kaplan-Meier survival analysis, the log-rank test was used, and survival curves were considered significantly different at $p < 0.05$. When appropriate, a one-way analysis of variance followed by a Newman-Keuls, multiple-comparison, post-hoc analysis was used to test for differences between samples, and data were considered significantly different at $p < 0.05$.

hSOD1 Copy Number Assessment

To determine whether the *SOD1G93A* copy number changed during the course of these experiments, we measured the copy number using a real-time PCR assay. Genomic DNA was extracted from tails with the use of a QIAGEN DNA extraction kit (QIAGEN, Valencia, CA). Brilliant II SYBR Green QPCR Master Mix reagent (0.15–20 ng; Stratagene, Santa Clara, CA) was used for real-time amplification of DNA. After heating at 50°C for 2 min and 95°C for 10 min, the DNA was amplified by 40 cycles of 95°C for 15 s and 60°C for 1 min, as suggested by Jackson Laboratories (Bar Harbor, ME). We found that the copy number did not change during the course of these experiments (Figure S2).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.08.004.

LICENSING INFORMATION

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